6/2/15

```
A. TE 10x Buffer Preparation
    EDTA - 0.188 g
    Trizma HCl - 0.288 g
    pH - 4.5 after mixing
    Desired pH of 7.5; added 1 M NaOH drop by drop until pH is 7.5

B. Zymoprep Yeast Plasmid Miniprep for HY6E1

C. Nanodrop to get nucleic acid concentrations of HY6E1 Digest
    Blank nanodrop with TE

D. HY6E1 Plasmid Digestion (creates Not 1 Cut)
    Not1HF (enzyme cut) - 0.5 uL
    HY6E1 (plasmid) - 5 uL
    Cutsmart (buffer) - 1 uL
    H2O - 3.5 uL
    overnight at 37C

E. Sequence and nanodrop Cadherin (competent cells)
    Cadherin 1 - 434.7
    Cadherin 2 - 166.1
```

6/3/15

```
A. Phosphatase
    1. Added 0.5 uL Antarctic Phosphatase to Not1-digested HY6E1 plasmid.
    *Phosphatase added to digest phosphate ends of the plasmid, so that the cut ends will not ligate back
immediately after.
    2. Incubated for 1 hour.

B. PCR Purification done according to protocol

C. Gibson for HY6E1 with cloning efficiency of 50-100 ng of vectors with 2-3 fold of excess inserts.
    1. Positive Control
    Gibson Master Mix - 5 uL
    Positive Mix - 5 uL

    2. Negative Control
    Vector - 4.25 uL (0.01132 pmol)
    Gibson Master Mix - 5 uL
    ddH2O - 0.75 uL

    3. Gibson Control
```

```
    Vector - 4.25 uL
    pTET alpha - 0.50 uL (0.035 pmol)
    tfBA1 - 0.20 uL
    Gibson Master Mix - 5 uL

    4. Incubate in thermocycler for 1 hour at 50C.
```

6/4/15

```
A. Colony PCR Cadherin (YPD + SD-Trp) according to protocol

B. Gibson HY6E1

C. PCR purify Not1 Digest according to protocol

D. Yeast Miniprep, nanodrop to get Digest concentration
    Note: Good range for nanodrop = 10-500, dilute to get more accurate reading

E. Redo colony PCR - E. Cadherin (YPD + SD-Trp) -- Failed. No bands at 400.
```

6/5/15

```
A. Redo Gibson because Colony PCR failed; vector = Not1-digested HY6E1

    1. Positive Control
    Gibson Master Mix - 5 uL
    Positive Control Mix - 5 uL

    2. Negative Control
    Vector - 2 uL
    Gibson Master Mix - 5 uL
    ddH2O - 3 uL

    3. Gibson Control
    Vector - 2 uL
    pTET-mf alpha - 1 uL
    tFBA1 - 0.25 uL
    ddH2O - 1.75 uL
    Gibson Master Mix - 5 uL

    4. Incubate in PCR incubator for 1 hour under "Gibson" pre-set. Lid temp at 95C.


B. Re-suspend primers in nm (found on the side of tube) x 10 uL ddH2O
```

```
    26.4 nm
    264 uL H2O
    10x stock, dilute 10-fold usually
    Send 1.75 mL 20x diluted to Quintara (87.5 uL primers, 1662.5 uL H2O)
```

6/8/15 - Day 1 Bootcamp

```
A. Cloning with Stanlee
    - Bar1 - sticks to protein's cell walls, protease enzyme
    - pTEF1(+Apa1)FW primer
    - pTEF1(+Xho1)RV primer
    - Followed Primer Design protocol

B. PCR for Constitutive Primers
        *Make sure to dilute primers to 10 uM from 100 uM stock primers
    Promoter and Bar1 both placed in PCR thermocycler overnight
    Primer stock solutions placed in Stanlee's freezer box
```

6/9/15 - Day 2 Bootcamp

```
A. Ran PCR products in gel - worked!
    1 uL loading dye (6x)
    5 uL PCR product
    Vortex
    (Try not to bubble when pipetting, results in loss of volume)
    Load at 5 uL into gel (35 uL tray filled with TAE buffer)

    Lane 1- gel ladder (7 uL)
    Lane 2- pTEF1 (5 uL)
    Lane 3- Bar1 (5 uL)
    Run at 100V!

B. Plasmid Digest
    Apa1 --promoter --Xho1-- Bar1 --Not1-- Plasmid --
        Notes: Always keep enzymes on ice!
    pTEF1 and Plasmid (PJW608) incubated at room temperature for an hour before adding the terminator primers
    Ran 3 PCR tubes (pTEF1, Bar1, PJW608 (plasmid)) in PCR incubator overnight
```

6/10/15 - Day 3 Bootcamp

```
A. Ran PCR products in gel
    Lane 1 - ladder (10 uL)
    Lane 2 - pTEF1  (40 uL)
    Lane 3 - pTEF1 (10 uL)
```

Lane 4 - Bar 1 (40 uL)
Lane 5 - Bar 1 (10 uL)
Lane 6 - PJW608 (36 uL)

B. Gel Extraction, followed QIAquick Gel Extraction Kit using a Microcentrifuge Protocol
Gel Weights
blank microcentrifuge tube: 1044.9 mg
pTEF1: 201.9 mg
Bar1: 355.1 mg
plasmid (PJW608) : 1243.0 mg

Step #2 - Add 3 volumes of buffer QG to 1 volume of gel (100 mg ~ 100 uL)
uL of buffer QG added in
pTEF1: 600 uL
Bar 1: 1050 uL
plasmid: 600 uL

Step #5 - Add 1 gel volume of isopropanol to sample and mix
uL of isopropanol added in
pTEF1: 200 uL
Bar 1: 355 uL
plasmid: 198 uL

Follow steps according to QIAquick Gel Extraction Kit Protocol

Step #13 - Elute DNA with 30 uL of water. Let sit for 1 minutes before centrifuging for 1 min.
Note: Place directly at the the center of the spin column cloth, or else it will not properly elute and there will be ethanol contamination!

After step #13, nanodrop and blank with ddH2O
Bar1 showed negative nucleic acid concentration (potential ethanol contamination), so used Eleanor's Bar 1 for ligation

C. Plasmid Ligation - using 2 kb
Vector DNA: 2 uL (50 ng)
Insert: 37.5 ng x 2 = 75 ng
75 ng / 15.44 ng/uL = 5 uL of Bar1
10 uL pTEF1
0 uL H2O
2 uL DNA Ligase buffer
1 uL DNA ligase
(good negative control would be if you don't put in the insert, ligate plasmid on its own = measures background)

D. E.Coli Transformation using New England BioLabs High Efficiency Transformation Protocol (C2987H/C2987I)
Notes:
- Competent E.Coli cells are in the -80 freezer, second shelf, middle box, 2nd row. The tubes are labeled with the E.Coli number.

```
        - When adding 5 uL of plasmid DNA to cell mixture, swirl with pipet tip. DON'T PIPET UP AND DOWN!
        - SOC media is easily contaminated because of high sugar content; be cautious to prevent
contamination!

E. E.Coli Plating
    Get warmed plates from incubator
    Labeled plates "pTEF1 - Bar 1 Ligation" + initials + date
    Plate #1 - 1x 100uL
    Plate #2 - 8x 100uL of resuspended ligated cells
        Pellet at 7-8000 rpm for 30 seconds
        Get 100 uL out, dump the rest and resuspend cells
        Put 100 uL of resuspended cells in the 8x labeled plate
    Shake plates with beads back and forth on table, not in the air
```

6/11/15 - Day 4 Bootcamp

```
A. Colony PCR for Screening E.coli
    1. Picked a single colony to plate, did this for 6 tubes
    2. PCR "Master Mix" (7x)
```

Material	Volume
2x GoTaq Green PCR Mix	70 uL
10 uM pTEF1 FW	7 uL
10 uM 93 REV	7 uL
H2O	21 uL
total	**50 uL**

```
    3. Added 15 uL of 7x Master Mix to 5 uL colony-filled tubes for total of 20 uL material.
    4. Put in thermocycler

B. E.Coli Gel PCR
    Lanes 2-7 - Stanlee and I's E.coli colony PCR - did not show bands

C. Culture Transformed E.coli in LB
```

6/12/15 - Day 5 Bootcamp

6/16/15

```
Bar-1 Constructs for iGEM Project: setting parameters for Bar-1 as T-reg analogs

A. Sent in CPL1(4)-Bar1 and CPL1(5)-Bar1 for sequencing to Quintara
    Came back with an empty plasmid PJW608
    Renamed tube "PJW608 Sequenced" and placed in Parts&Plasmids 2014 box
```

6/17/15

```
A. Promoter PCR
    1. Followed Promoter PCR Reaction Protocol to re-PCR all pTEF1 mutants

    Tube Label          Template    Promoter    Primer
    A                   pGEM 17     pTEF1       113 FW/114 RV
    B                   pGEM 19     M6          116 FW/114 RV
    C                   pGEM 20     M7          117 FW/114 RV
    D                   pGEM 16     M10         118 FW/114 RV

    2. Ran promoters PCR products in gel - A and D did not work, B and C did
```

insert gel image here

6/18/15

```
A. Check sequencing for "pGEM13+rtTA3" and "pGEM13+rtTA4" -- sequences matched and tubes contain pGEM18.
    Relabeled tube "pGEM18 sequenced" and placed in Parts&Plasmids box

B. Ran gel for PCR products (10 uL of loading dye/50 uL of product)

    1       2       3       4       5       6           7       8       9
ladder    Bar1A   Bar1B     x    pGEM13-1  pGEM13-2     x     A redo   B redo


C. Isolate pBAR1 from Cadherin
    1. Followed Zymolase Yeast Plasmid Miniprep Protocol

D. PCR pBAR1 under Phusion protocol on PCR machine
```

Material	Volume
Template (Zymolase Cadherin)	5 uL
primer 119	2.5 uL
primer 120	2.5 uL
2x Fusion Master Mix	25 uL

H2O	15 uL
total	**50 uL**

Insert image here

6/19/15

```
A. PCR purified pBAR1, followed PCR Purification Protocol

B. Ran gel to verify pBAR1 presence
    Lane 1 - DNA ladder
    Lane 2 - pBAR1-A
    Lane 3 - pBAR1-B
    none of the pBAR1's showed up in gel, very streaky
    Kara thinks it might be because the promoters are too large to show up on the gel

C. Digested pJW608 with Xho1 and Not1

D. Digested Bar 1 with Xho1 and Not1

E. Re-PCR pTEF1 with EC template, run in gel
```

Insert image here

6/22/15

```
A. Ran pTEF1 PCR product in gel, worked!

B. Gel Purification of Digested Bar1 and Plasmid
    Used QIAquick PCR Purification Kit
        1:5 Buffer PB:digest
        pJW608 digested (30 uL), added 6 uL Buffer PB
        Bar1 digested (56 uL), added 11 uL Buffer PB

C. Ligation
```

Insert image here

Material	Negative Control	Control
PJW608	2 uL	2 uL
BAR1	0 uL	1 uL

Ligase	1 uL	1 uL
Ligase Buffer	1 uL	1 uL
H2O	16 uL	15 uL
total	**16 uL**	**15 uL**

6/23/15

```
A. Transformed ligated PJW608-BAR1 and Negative Control into NEB-5alpha E.coli cells.

B. Plated transformed cells.
    Negative control had more colonies than the control.
```

6/24/15

```
A. Phosphatase (Antarctic)-treated PJW608 Xho1,Not1-digested plasmid
    0.5 uL phosphatase
    Incubated at 37C for 1.5 hr
    Heat inactivated at 80C for 20 mins

B. Colony PCR transformed cells using Colony PCR Protocol.

C. Ran colony PCR products in gel.
    Lane 1 - ladder (10 uL)
    Lanes 2-7 - Colony PCR control products (10 uL)
    Lane 8 - ladder (10 uL)
    Lane 9-13 - Colony PCR negative control (10 uL)
    Lane 14 - ladder (10 uL)
```

6/25/15

```
A. Successful gel colony PCR were #3,4,6 control products

B. Miniprepped successful PCR colony products and nanodropped
```

Insert gel image here

6/26/15

```
Out of lab
```

6/29/15

```
A. Ran gel to confirm pTEF1

B. Digested pTEF promoters
    - 5 uL cutsmart
    - 0.5 uL Apa1 --> incubate at room temp for 1 hour
    - 0.5 uL Xho1 --> incubate at 37C overnight

pTEF1 and pTEF1(m3) gel runs successful ~400 bp.

- Lane 1: DNA Ladder
- Lane 3: pTEF1
- Lane 4: pTEF1(m3)
```

Insert gel image here

6/30/15

```
A. Digested pTEF1(m3)
    - 0.5 uL Apa1 --> incubate at room temp for 1 hour
    - 0.5 uL Xho1 --> incubate at 37C for 1 hour
B. Digested plasmid
    - 0.5 uL Apa1 --> incubate at room temp for 1 hour
    - 0.5 uL Not1 --> incubate at 37C for 1 hour (done at 12pm)
```

7/1/15

```
A. Ran 1% agarose gel with all PTEF1 mutants
    -Everything worked except for pTEF1

B. Re-PCR pTEF 1 using 2 templates: one from pGEM17, one from EC Bootcamp template
    -Neither had successful correct bands
    -Redo PCR of pTEF1 again
```

Insert image here

7/2/15

```
A. Ran 1% agarose gel for the pTEF1 PCR products from the previous day.
    -Again, gel did not show proper bands
    -Rethink source of pTEF1
```

B. Ran 1% agarose gel for other pTEF1 mutants (m3, m6, m7, m10) and BAR1
    - Lane 1: DNA ladder
    - Lane 3: BAR1
    - Lane 5-6: pTEF1 m3
    - Lane 7-8: pTEF1 m6
    - Lane 9-10: pTEF1 m7
    - Lane 11-12: pTEF1 m10
    - Lane 14: DNA ladder

    pTEF gel bands successful at ~400 bp. However, plasmid does not show presence of BAR1 on gel.
        Troubleshoot: Digested plasmid used to run gel for BAR1 was digested with the wrong cut enzymes.

C. Digested PJW608 + BAR1 with Apa1 and Xho1 cut sites.

D. Gel extracted pTEF1 gel products (Measurements shown in Table 1 below)

E. Yeast Genomic DNA Purification Kit using the Amresco Protocol
    -Harvested yeast cells from solid media by restreaking in new YPD plate --> culture over weekend

pTEF Mutant	Gel weight (mg)	Buffer QG Added (3mL:1)	Nanodrop concentration (ng/uL)
pTEF1 m3	647	1950	79.93
pTEF1 m6	569	1710	63.22
pTEF1 m7	387	1160	74.01
pTEF1 m10	808	2430	59.07

To Do:
    - Re-do Yeast Genomic DNA Purification Kit with the restreaked Cadherin
    - Gel extract digested plasmid with BAR1
    - Ligate PJW608/BAR1 and pTEF1 mutants
    - AMPLIFY PTEF1!!! (PCR/gel extract/ligate) try with GoTaq, isolate pTEF1 from TEF1 (can try to isolate from yeast genomic DNA purification kit)

7/6/15

A. Re-did Yeast Genomic DNA Purification with the newly streaked Cadherin EBY100 plate
    Nanodrop concentration: 5.69 ng/uL

B. Grow overnight culture of EBY100 in YPD (5 ml)

C. PCR yeast DNA plasmid to amplify pTEF1 (113 FW/114 RV) and pBAR1 (119 FW/120 RV) under GoTaq protocol.

Materials	pTEF1	pBAR1
FW primer	2.5 uL	2.5 uL
RV primer	2.5 uL	2.5 uL
GoTaq Buffer	25 uL	25 uL
ddH2O	8.5 uL	8.5 uL
DMSO	1.5 uL	1.5 uL

```
D. Ran PCR products in gel, bands seen at:
    350 bp - pBAR1
    400 bp - pTEF1
    9 kb - BAR1

E. Transformed pTEF1 mutants + negative control into E.coli competent cells.
```

7/7/15

```
A. Colony PCRd transformation products from previous day
    Master Mix - 5x for each promoter
        2x Go Tag - 50 uL
        10 uM FW primer - 5 uL
        10 uM RV primer - 5 uL
        water - 15 uL

        m3 - 115 FW/114 RV
        m6 - 116 FW/114 RV
        m7 - 117 FW/114 RV
        m10 - 118 FW/114 RV

B. Ran colony PCR products in 1% agarose gel and set up overnight liquid cultures for positive colonies in LB-
Carb (for miniprep next day).
    Lane 1 - ladder
    Lanes 2-4 - Negative control colonies 1-3
    Lanes 5-7 - m3 colonies 2-4
    Lanes 8-11 - m6 colonies 1-4
    Lanes 12-14 - m7 colonies 2-4
    Lanes 15-17 - m10 colonies 1-3
    Lane 18 - ladder

C. Made more 1% agarose. Actual amounts: 5.034 g/500 mL 1x TAE buffer
    Microwaved for 5 minutes, let cool before pouring

D. Ran BAR1 1.5% agarose gel for 45 minutes for gel extraction -- band seen at 9 kb, but looks uncut
```

```
    Send in pGEM45 for sequencing to see if plasmid is actually cut

E. Digest pTEF1 and pBAR1 overnight
    5 uL Cutsmart on ice
    0.5 uL Apa1
    Incubate at room temp for 1 hr.
    0.5 uL Xho1
    let digest overnight

F. pGEM45 Transformation into E.coli
    C2987 cells - 25 uL
    pGEM45 - 0.5 uL
    30 mins ice
    45 sec heat shock at 42C
    2 min ice
    250 uL SOC
    Incubate 1 hr at 37
    Plate on LB-Carb (100 uL) overnight
```

TO DO:

-Gel extract pTEF1 and pBAR1 from digestion products
-Colony PCR transformed cells with pGEM45
-overnight liquid culture positive colonies in LB-Carb?
-PCR BAR1 from pGEM45
-Gel extract BAR1+pJW608
-pTEF1 ligation
-pBAR1 ligation
-pTEF mutants ligation (if colony PCR does not work)
-Transform ligation products into E.coli

7/8/15

```
A. Ran pTEF1 and pBAR1 digests on 1% agarose gel.

B. Gel extracted pBAR1 and pTEF1 using QIA Gel Extraction Protocol.
```

Extracted Bands	Gel weight (mg)	Buffer QG Added (3mL:1)	Nanodrop concentration (ng/uL)
pTEF1	301	903	41.38
pBAR1	238	714	29.88

```
C. Miniprepped overnight positive cultures of E.Coli pTEF mutant transformations.
```

Nanodrop concentrations:
m3 colony 3 - 305.0 ng/uL*
m3 colony 4 - 15.069
m6 colony 3 - 471.1*
m6 colony 4 - 418.9
m7 colony 2 - 360.4
m7 colony 3 - 378.4*
m10 colony 1 - 477.7
m10 colony 2 - 481.1*

D. *Starred miniprepped colonies sent in for sequencing with "BO.R1" primer.

E. Set up liquid overnight cultures of CB008DB for yeast promoter transformation in 5mL YPD.

F. Set up liquid overnight culture of transformed pGEM45 + CB008DB (to obtain more BAR1+pJW608 plasmid) in LB-Carb.

7/9/15

A. Checked sequencing for pGEM45 + pTEF1 mutants
1. m3, m6 and m7 showed no promoter sequences -- re-ligate pTEF1 mutants into
2. m10 contained promoter and BAR1 sequence -- move on to yeast transformation! Relabeled tube "pGEM 46"
(pGEM46 = pJW608 + BAR1 + pTEF1 m10)

B. Sent other positive colony PCR pTEF1 mutants for sequencing to see if they have the promoter inserts

C. Yeast Transformation
1. Diluted overnight culture of CB008DB
9.5 mL YPD
500 uL CB008DB culture
Incubate at 30C for 3 hours
2. Digested plasmid
2000 ng DNA - 4.16 uL m10 plasmid
.5 uL PME1
2.5 uL CutSmart
18 uL water
Incubate 37C for 1 hour
3. Boil salmon sperm 30 minutes prior to transformation

D. Miniprep overnight culture of pGEM45
1. Sent 5uL to sequencing - sequencing correct!

E. Digested pGEM45 with Apa1 and Xho1
Phosphatase treated with Antarctic phosphatase (0.5 uL/vortex)
1 hour at 37C
20 mins at 65C

F. Ran pGEM45 digestion in 1.5% agarose for 20 minutes, saw presence of BAR1 around 1kb (INSERT GEL IMAGE)

G. Gel extracted pGEM45 digestion to be used for pTEF1 and pBAR1 ligations for tomorrow

7/10/15

A. Sequencing results
    pGEM45 - contains BAR1
    m6 + m7 - contain BAR1, but do not have promoters

B. Ligated pTEF1 into pGEM45
    Ligated pBAR1 into pGEM45

C. Miniprepped m3 overnight cultures for E.Coli transformation. Send miniprepped m3 for sequencing

D. Since m6 and m7 didn't work, need to re-digest

7/14/15

A. Run Colony PCR 1, 2 and 5 in gel *insert gel image*
    Colony 1 showed the brightest bands

B. Since pTEF1 m3, m6 and m7 ligations to pGEM did not work..
    1. Re-digest m3, m6, m7 with Apa1 and Xho1 cut sites
    2. PCR purify digests, nanodrop
        m3 - 6.12 ng/uL
        m6 - 15.5 ng/uL
        m7 - 7.56 ng/uL
    Nanodrop concentrations are very low levels of DNA, start new PCR of m3, m6, m7

C. Transformed pTEF1 and pBAR1 ligation into E.Coli, plated in LB-Carb at 37C overnight

D. Re-streaked patch plate of yeast transformed pGEM46 onto YPD plate
Incubate overnight at 30C

E. Overnight cultured colony 1 of yeast transformed pGEM46 patch plate for yGEM glycerol stock!

7/15/15

A. Dilute 1:20 o/n culture of yeast transformed pGEM46 with YPD
     250 uL o/n pGEM46 in yeast
     4.75 mL YPD
     Wait 2 hours
     Place in glycerol stock cryovial -- YGEM!!

```
B. Run pTEF1 m3, m6 and m7 PCR products in 1% agarose gel for 30 mins
    m3 and m6 showed clear bands at 450bp, no bands in m7
```

7/16/15

```
A. Digested pTEF1 m3 and m6 with Apa1 and Xho1

B. Gel purified pTEF1 m3 and pTEF1 m6 digests
    Nanodrop concentrations:
    m3 - 32.58
    m6 - 34.99
```

7/17/15

```
A. Gel extracted plasmids (pGEM1, 2, 45)
```

Plasmid	Gel weight (mg)	Buffer QG Added (3mL:1)	Nanodrop concentration (ng/uL)
pGEM1	196	588	
pGEM2	257	771	27.45
pGEM45	226	678	45.34

7/20/15

```
A. PCR m3, m6, m7 (because the gel extractions did not yield good nanodrop concentration amounts)

B. Ran promoters in 1% gel for 25 minutes -- good bands at ~450bp

C. Digested m3, m6, m7 with Apa1 at room temperature for 2 hours
    With Xho1 at 37C for 3 hours
```

7/21/15

```
A. Gel extracted pTEF1 m3, m6 m7
```

pTEF Mutant	Gel weight (mg)	Buffer QG Added (3mL:1)	Nanodrop concentration (ng/uL)

pTEF1 m3	170	510	
pTEF1 m6	220	660	
pTEF1 m7	289	867	

```
B. 10-fold dilution of promoters for ligation
    Ligated pTEF1 m3, m6, m7 to pGEM45

C. Yeast Genomic DNA Purification of EBY100 to obtain pBAR1
    Nanodrop concentration:

D. Transformed pGEM45+pTEF1 mutant ligations into E. Coli.
```

7/22/15

```
A. Colony PCR'd pTEF1 m3, m6, m7 E. Coli transformations.
    Lane 1 - ladder
    Lane 2-4 - m3
    Lane 5-8 - m6
    Lane 9-12 - m7
    Lane 13 - ladder
    All showed bands corresponding with lengths ~450 -- correct!

B. Back-up plan in case colony PCR/minipreps don't work
    Phosphatase treat pGEM45 gel extraction for new ligations
    Incubate overnight at 37C
    Heat inactivate at 70C for 15 minutes

C. Digest pBAR1 and pTEF1 with Apa1 and Xho1 overnight.
```

7/23/15

```
A. Overnight culture positives for colony PCR in 5 mL LB-Carb.
    pTEF1 m3 - colony 1 & 3
    pTEF1 m6 - colony 1 & 2
    pTEF1 m7 - colony 1 & 2
    Incubate at 37C

B. Back-up plan in case promoter colony PCR/minipreps don't work (or if we somehow get false positives again).
    2. Re-ligate phosphated treated pGEM45 to m3, m6, and m7 gel extracts

C. Ligate pBAR1 to pGEM1 and pGEM2
        Diluted pBAR1 10-fold (1 uL pBAR1, 9 uL Nuclease-free H2O)
    1. pGEM1 + pBAR1 ligation
```

```
        1 uL diluted pGEM1 (concentration: 239.9 ng/uL)
        1 uL 10 xdiluted pBAR1 (concentration: 73.21 ng/uL)
        2 uL T4 Ligase Buffer
        1 uL Ligase
        15 uL Nuclease-free water

    2. pGEM2 + pBAR2 ligation
        2 uL pGEM2 (concentration: 27.45 ng/uL)
        1 uL 10x diluted pBAR1 (concentration: 73.21 ng/uL)
        2 uL T4 Ligase Buffer
        1 uL Ligase
        14 uL Nuclease-free water
    2 hour incubation at room temperature

D. PCR pTEF1 with EC/SQ backbone for template
    Re-diluted new 113 primers in case primers were issue
    Run in 1% agarose gel for 20 minutes -- no bands shown
    **Since pTEF1 shows the same GFP expression as m10, we are foregoing the use of pTEF1
```

7/24/15

```
A. Miniprepped overnight cultures for pTEF1 mutant colony PCR.
    All sent to Quintara for sequencing

B. Colony PCR backup pTEF1 mutant and pBAR1 transformations.
    Ran in 1% agarose gel for 30 minutes
    Kept positives in freezer for weekend, will overnight culture on Monday
```

7/27/15

```
A. pTEF1 mutant transformations in pGEM45 sequencing incorrect.
    Re-ligated and re-transformed.
    Transformation plates did not have any colonies -- will Gibson to see if that works better.
```

7/28/15

```
A. Digested pGEM45 with Xho1 only for Gibson to cut open the plasmid.

B. PCR pTEF1, m3, m6 and m7 with new primers with overhangs for Gibson Assembly
```

pTEF Mutant	Forward primer	Reverse primer
pTEF1	164	165

pTEF1 m3	166	165
pTEF1 m6	167	165
pTEF1 m7	168	165

7/29/15

```
A. Sequencing of pBAR1 + pGEM1/2 came back correctly! Labeled tube as "pGEM51."

B. Overnight culture CB008DB in 30C for pGEM51 yeast transformation.

B. Gel extracted PCR products from yesterday for Gibson.
```

pTEF Mutant	Gel weight (mg)	Buffer QG Added (3mL:1)	Nanodrop concentration (ng/uL)
pTEF1	98.4	295	
pTEF1 m3	123.7	369	
pTEF1 m6	120.1	360	
pTEF1 m7	113.6	340	

7/30/15

```
A. PME-digest pGEM51 - 0.5 uL/ug

B. Boiled salmon sperm DNA
    95C - 10 minutes
    ice - 20 minutes

C. Yeast transformed pGEM51, plated on SD-Ura
    Incubated at 30C for 72 hours.
```

7/31/15

```
A. Gibson Assembly gel extractions of pTEF1 mutants to pGEM45.
B. Remade 10x TE Buffer, 1M LiOAc, and 0.1M LiOAC in 1x TE Buffer
    Followed Jeffrey's recipe for 10x TE Buffer and 1M LiOAc

    For 1M LiOAc - 10.202 g LiOAc/100 mL H2O
        Vacuum filter
```

```
    For TE Buffer 10x
        .576 Trizma HCl/100 mL ddH2O
        .372 g EDTA/100 mL ddH2O
        Shake
        pH 4.5
        Added 1.2 mL 1M NaOH until pH is 7.5
        Vacuum filter

    For 0.1M LiOAc in 1x TE:
        ddH2O - 40 mL
        1M LiOAc - 5 mL
        10x TE Buffer - 5 mL
```

8/3/15

```
A. Patch plate for pGEM51 yeast transformation

B. Colony PCR PGEM51 yeast transformation
    Master Mix 6x
        2x GoTaq - 60 uL
        158 FW - 6 uL
        159 RV - 6 uL
        water - 18 uL
        boiled yeast - 5 uL each for 30 uL
        Total - 120 uL

C. Gibson Assembly of all pTEF1 mutants had colonies on plates, but no colonies at all on the positive
control, many colonies on negative control plate
    Re-digested PGEM45 for Gibson Assembly
    pGEM 45 - 13 uL
    Apa1 - 1 uL
    Cutsmart - 2 uL
    ddH2O - 3 uL
    Incubate room temp for at least 1 hour

    Xho 1 - 1 uL
    Incubate 37C for at least 1 hour

D. Colony PCR Gibson Assemblies
    Master Mix 5x
        2x GoTaq - 50 uL
        promoter FW primer - 5 uL
        Bar1 RV primer (93) - 5 uL
        water - 15 uL
        colonies - 5 uL each
```

```
E. Overnight cultured the following for initial flow cytometry:
    CB008DB - yeast for negative control
    pGEM43 - pAga1-GFP
    yGEM128 - pTEF1 m10-BAR1
```

8/4/15

```
A. Ran colony PCR of Gibson on 1% agarose gel for 25 minutes -

B. Re-colony PCR of pGEM51 yeast transformation
    Took colonies from patch plate
    158 FW/159 RV primers
    Ran on 1% agarose gel for 25 minutes -- showed bands at 350 bp where pBAR1 should be! --> yGEM 132!!!
    o/n culture yGEM132 to make glycerol stock tomorrow

C. Diluted overnight cultures for flow cytometry
    Took OD measurements
    Set up 96 well-plate with triplicates of CBD008DB,pAga1-GFP, pTEF1 m10-BAR1, pAga1-GFP+pTEF1 m10-BAR1
    Incubate-shake at 30C for 3 hours
    Induced with 0, 0.5, 1, 10, 1000 nM of alpha factor
```

8/5/15

```
A. Made glycerol stock of YGEM132
    50% glycerol - 420 uL
    yGEM132 overnight culture - 350 uL
    snap freeze
    Put in -80C freezer

B. Gibson Assembly of pTEF1 mutants to pGEM45
    PCR Purified Apa1/Xho1-digested pGEM45

C. E. Coli transformation of Gibson assemblies into LB-Carb plates
    All pTEF1 mutant Gibson plates had colonies, very few colonies on negative plate, presence of colonies on
positive plate
```

8/6/15

```
A. Colony PCR pTEF Gibson E.Coli transformations

B. Flow Cytometry for CB008DB, YGEM 34, 128, 129, 130
```

8/7/15

A. Miniprepped overnight cultures of the transformed Gibson pTEF1 mutants.
    pTEF1 - 1,3
    m3 -
All had high nanodrop concentrations
Sent to sequencing
    pTEF1 - sequencing correct!
    pTEF1B - sequencing correct!
    m3A - sequencing correct with promoter!
    m3B - sequencing correct with promoter!
    m6A - sequencing correct!
    m6B - sequencing correct!
    m7A - sequencing correct!
    m7B - sequencing correct!

B. Yeast Transformations to have blue label in Bar1 strains
    yGEM128 (pTEF1m10-Bar1) + pGEM34 (pNH604 pAGA1 BFP TRP)
    CB008DB + pGEM34

    1.PME1-digested pGEM34
        pGEM34 - 3 uL
        PME1 - 0.5
        CutSmart - 1 uL
        H2O - 5.5 uL
        Total - 10 uL
        Incubate at 37 for 3 hours

    2.Boil ss for 10 minutes
        Put on ice for 20 minutes

C. E.Coli transformation to make more pGEM34

8/10/15

A. Re-labeled all pTEF1 mutant minipreps
    pTEF1 - pGEM58
    pTEF1 m3 - pGEM59
    pTEF1 m6 - pGEM60
    pTEF1 m7 - pGEM61

B. Yeast transformations of pGEMs from A
    1. Diluted CB008DB
        500 uL incubated yeast cells
        9.5 mL YPD
        Incubate at 30 for 3 hours
    2. Digested all plasmids
        2000 ng plasmid DNA

```
        .5 uL PME1
        2.5 uL Cutsmart
        Add H2O to bring total volume to 20 uL
        Incubate at 37 for 2 hrs
    3. Boil salmon sperm 30 mins before yeast transforming
        95C for 10 mins, ice for 20 mins
    4. Yeast transformation protocol

C. Made new PEG because Josh left it out
```

8/11/15

```
A. Flow cytometry
    CB008DB A1-A6, B1-B6, C1-C6
    YGEM 34 (pAga1-GFP) A7-A12, C7-C12
    YGEM 132 (pBAR1-GFP)
    YGEM 134 (pTEF1m10-Bar1-pAga1-BFP)
```

8/13/15

```
A. Miniprepped positive o/n cpultures of pBar1(synthesized)-PGEM1 E.Coli transformations
    Sent to sequencing

B. Colony PCR of pTEF1 mutant-Bar1 yeast transformations (NatMX)
    91 - Nat MX integration FW primer
    87 - 600 integration RV primer
```

8/14/15

```
A. Gibson Assembly of pTEF1m10-BFP
    1. PCR pTEF1m10
        template DNA pGEM46 - 0.5 uL
        171 FW - 2.5 uL
        172 RV - 2.5 uL
        Phusion 2x MM - 25 uL
        Water - 19.5 uL
        Total - 50 uL
    2. Digested pGEM34 with Apa1 and Xho1
    3. Gel extracted pGEM34 digest and pTEF1m10 PCR product
    4. Gibson Assembly and E.Coli transformation
```

8/17/15

```
A. Colony PCR pTEF1m10-BFP Gibson Assembly transformation
    5 colonies - 6x MM
    GoTaq 2x - 60 uL
    118 m10 FW - 6 uL
    97 BFP RV - 6 uL
    ddH2O - 18 uL
    Run under GoTaq protocol

B. Ran colony PCR in 1% agarose gel for 25 minutes, all bands were ~900bp which was the correct length
    O/n cultured colonies 2 and 3
```

8/18/15

```
A. Miniprepped positive cultures of PTEF1m10-BFP transformations
```

8/19/15

```
A. pTEF1m10-BFP A sequencing came back correct, B was not correct --> renamed pGEM63
    1. PME1 digest of PGEM63
        2 uL pGEM63 template DNA
        .25 uL PME1 digest
        2.5 uL Cutsmart
        add ddH2O until 20 uL total
    2. Dilute yGEM 143-146
        .25 mL yeast culture
        4.75 YPD
    3.Yeast transformed in SD-Trp of PTEF1m10-BFP into Bar1 strains:
        yGEM 143
        yGEM 144
        yGEM 145
        yGEM 146
        CB008DB

B. Flow Cytometry
    Diluted DB, yGEM 147 and 155 to 0.015 dilution factor for 1.5 mL media
    Induced with 0, 0.3, 0.6, 3, 6, 9 ug/uL alpha factor
    Took timepoint plates at hour 0, 1.5, 3, 5, 8
    Ran flow cytometry
```

8/21/15

```
A. Colony PCR yGEM 143/144/145-pTEF1m10-BFP yeast transformations
    (yGEM 146 + pTEF1m10-BFP did not have colonies)
    20x MM
```

```
    2x GoTaq MM - 200 uL
    160 Trp-integration FW primer - 20 uL
    87 600 integration RV primer - 20 uL
    ddH2O - 60 uL

B. Ran colony PCR in 1% agarose gel
```